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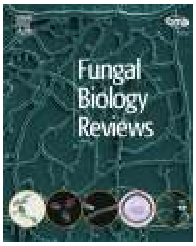

Review

Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact

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Keywords:
Botryosphaeria
Botryosphaeriaceae
Emerging tree diseases
Endophyte
Latent pathogen
Quarantine
Tree health

ABSTRACT

In many respects, the ecology of members of the Botryosphaeriaceae compare to general patterns observed for the collective of endophytes of woody plants. These include high levels of diversity, horizontal transmission a spatial structure and a continuum of levels of host affinity from specific to very broad. Some members of the Botryosphaeriaceae are, however, among the most aggressive pathogens in the assemblages of common endophytic fungi, often killing large parts of their host, following physical damage or general stress on the host (and over large areas). Their wide occurrence, the latent phase which can be overlooked by quarantine, and their ability to rapidly cause disease when their hosts are under stress, make these fungi a significant threat to agricultural, plantation and native forest ecosystems alike. This is especially relevant under emerging conditions of dramatic climate change that increases stress on plant communities. It is, therefore, important to maximize our understanding of the ecology and pathology of the Botryosphaeriaceae, particularly as it relates to their endophytic nature, species richness, host switching ability and the host-fungus-environment interaction.

1. Introduction

Members of the fungal family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes), were first described in the 1820's as species of *Sphaeria* (Fries) (for reviews see Crous *et al.* 2006; Schoch *et al.* 2006). The genus *Botryosphaeria* was only established in 1863. At the time, these Ascomycetes with their sphaerical ascomata and bi-tinucate asci were noticed as saprophytes fruiting on dead tissue of woody plants. Later studies have focused on *Botryosphaeria* and its anamorph genera as pathogens, especially in agricultural, nursery and plantation forestry situations (Bega *et al.* 1978; Brown & Britton 1986; Michailides 1991; Swart & Wingfield 1991; von Arx 1987). Currently, more than 2000 names are linked to this family, including teleomorph and anamorph states of which *Diplodia*, *Botryosphaeria*, *Fusicoccum*, *Dothiorella*, *Lasiodiplodia* and *Sphaeropsis* contain the most species.

Studies on various biological aspects of the Botryosphaeriaceae, including their endophytic nature, have suffered from the taxonomic complexities and confusions that have plagued the group. Recent advances in DNA- based molecular techniques have begun to provide efficient tools to characterize the presence and identity of species of the Botryosphaeriaceae. Studies applying these tools are revealing significantly greater diversity on some hosts than was previously realized. Recently, the group has also been separated into numerous

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doi:10.1016/j.fbr.2007.06.002

distinct genera (which were all previously linked under the teleomorph genus *Botryosphaeria*) (Crous *et al*. 2006). This has necessitated the difficult and confusing process of numerous name changes, yet it has provided a more natural classification for this group. This should allow for a more stable and accurate taxonomic framework in future and this will strongly influence our understanding of the ecology of the Botryosphaeriaceae.

Although it has been known for some time that Botryosphaeriaceae can infect through natural openings of healthy plants, it was only in the late 1980's that they were recognized as endophytes. The important forest pathogen *Diplodia pinea* (as *Sphaeropsis sapinea*) was isolated from the stems and xylem of *Pinus* (Petrini & Fisher 1988). Soon afterwards a number of *Fusicoccum*, *Neofusicoccum*, *Pseudofusicoccum* (all known at the time as *Dothiorella* spp). and *Lasiodiplodia theobromae* were isolated from healthy mango plant parts (Johnson *et al*. 1992). Since then a number of other species have been isolated as endophytes. Based on the wide taxonomic distribution and high frequency of endophytic infection on various hosts for those species examined to date for endophytism, it is now thought that most, if not all Botrysphaeriaceae, might have an endophytic phase.

Diseases caused by Botryosphaeriaceae mostly follow the onset of stress due to factors other than the Botryosphaeriaceae infection itself (Blodgett & Stanosz 1995; Schoeneweiss 1981; Swart & Wingfield 1991). These disease symptoms can, however, developed rapidly and cause extensive losses over large areas, if the agent of stress is widespread. Climate change models predict extreme weather conditions, such as unpredictable rainfall, lower or higher rainfall in different areas, extreme heat or cold, and more (Coakley *et al*. 1999). These factors, together with additional biological pressure from pathogens and pests expanding their geographic ranges, are all elements that would favor the development of Botryosphaeriaceae-related diseases (Desprez-Loustau *et al*. 2006). It is thus critical to better understand the ecological role, pathogenicity, diversity, host-pathogen-environment interaction and human mediated movement to define and address the threat that they might pose under such conditions.

The Botryosphaeriaceae represent a diverse and often prominent component of many endophytic communities. Yet, despite this fact, and the long standing recognition of their economic importance, their presence and ecological role in native plant communities is poorly studied. Factors influencing pathogenicity are also not always clear, and consequently their importance as quarantine fungi is poorly defined. Taxonomic difficulties make addressing these questions, and interpreting earlier work, particularly difficult. Here we review the taxonomic status, tools for isolation and identification, ecology, pathogenicity and endophytic nature of the Botryosphaeriaceae, and compare these to what is known about other fungal endophytes of woody plants. We attempt to identify general patterns from these data which will enable us to better understand the ecology of these fungi, particularly in their endophytic manifestation, and to define avenues for future research.

2. Terminology

The use of the term 'endophyte' can be controversial when it refers to the ecological role of the organism, particularly in describing mutualists vs. latent pathogens. Some Botryosphaeriaceae are clearly known as pathogens, and could thus be described as latent pathogens. It would be a mistake, however, to refer to the group as latent pathogens. Even for many of the well- studied pathogens, pathogenicity might vary greatly in different environments and little is known about their ecology in native ecosystems. For most of the species, nothing is known regarding their ecology. This complicates the use of specific terms referring to ecology. In this paper we use the term 'endophyte' to refer to the Botryosphaeriaceae in a general sense and as it relates to their presence within healthy plant tissue, rather than on the surface, without necessarily implying any specific ecological role (see also Saikkonen *et al*. 1998; Stone & Petrini 1997).

3. Diversity, taxonomy and identification in the Botryosphaeriaceae

The Botryosphaeriaceae (the sole family in the Botryosphaeriales (Schoch *et al*. 2006); Figs 1–17) represent a diverse family including more than 2000 taxa (Index fungorum; http://www.indexfungorum.org). The taxonomy of genera and species in the Botryosphaeriaceae has, however, been confused for a long time. This frustrates the interpretation of older literature on the endophytic nature, pathogenicity and host association of Botryosphaeriaceae. In recent years, mostly due to the availability of DNA- based molecular tools, a more robust taxonomy has emerged for this group. Here we explore the history and current understanding of the taxonomy of the Botryosphaeriaceae. We also consider tools used for identification and that provide a framework in order to interpret past research, and to undertake future work regarding the ecology of these fungi.

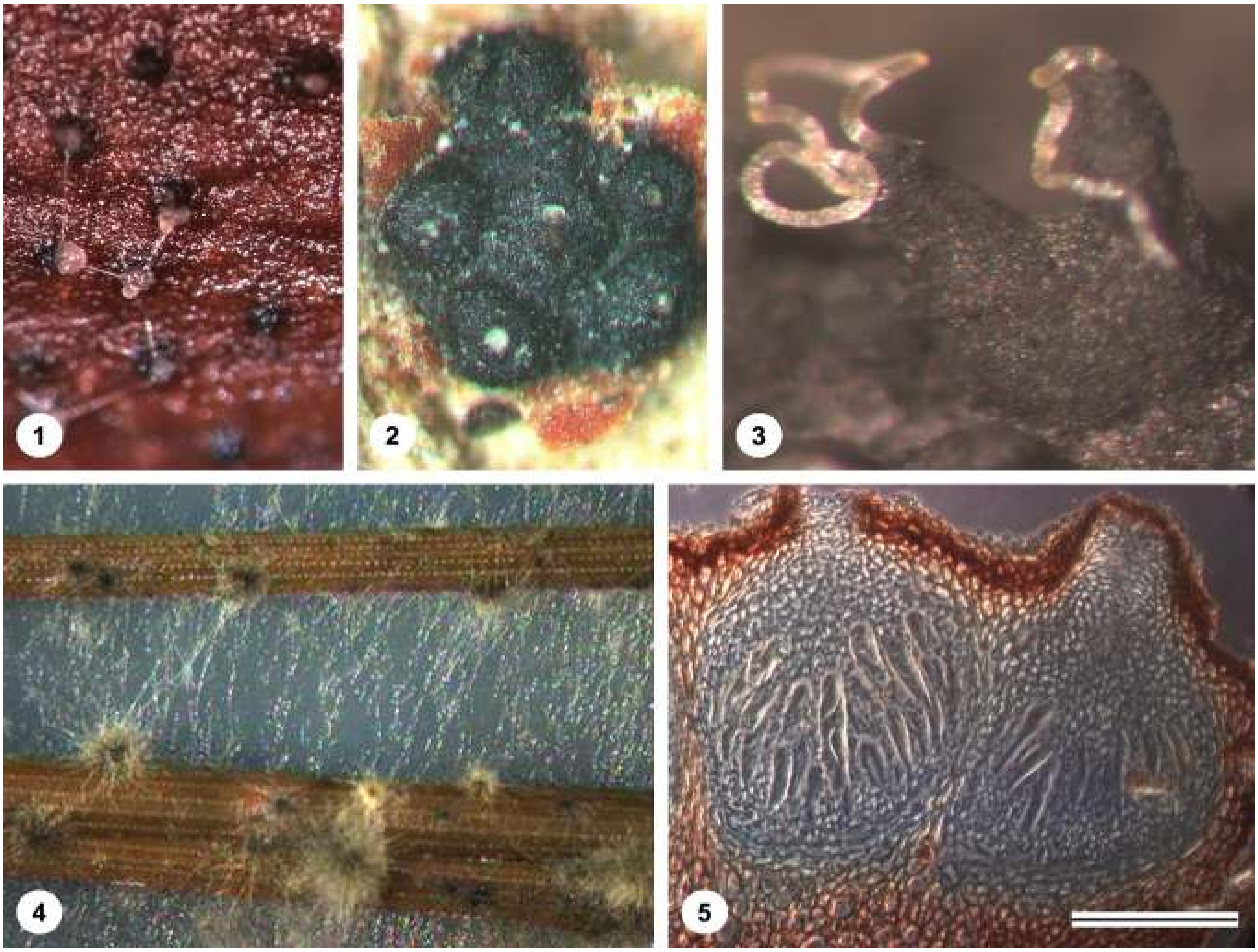
Taxonomy

In an important and widely used revision of the genus *Botryosphaeria*, von Arx and Müller (1954) reduced a large number of species to synonymy, particularly using the names *B. quercuum* and *B. dothidea*. As a result, many distinct species have been treated under the name *B. dothidea*, while some of its synonyms (e.g. *B. ribis* and *B. berengeriana*) have been used inconsistently in the literature. This problem endured for many years and it has raised substantial confusion, as well as misinterpretation of the literature. Slippers *et al*. (2004a) epitypified *B. dothidea* and characterized the taxa formerly treated under this name, including *N. ribis* (= *B. ribis*) and *N. parvum* (= *B. parvum*). However, the confusion of names used under these and other *Botryosphaeria* binomials in past studies remains difficult to interpret, and particularly affects the older literature related to the endophytic nature of these fungi. This literature must be interpreted with caution when analyzing patterns of diversity, distribution and host association.

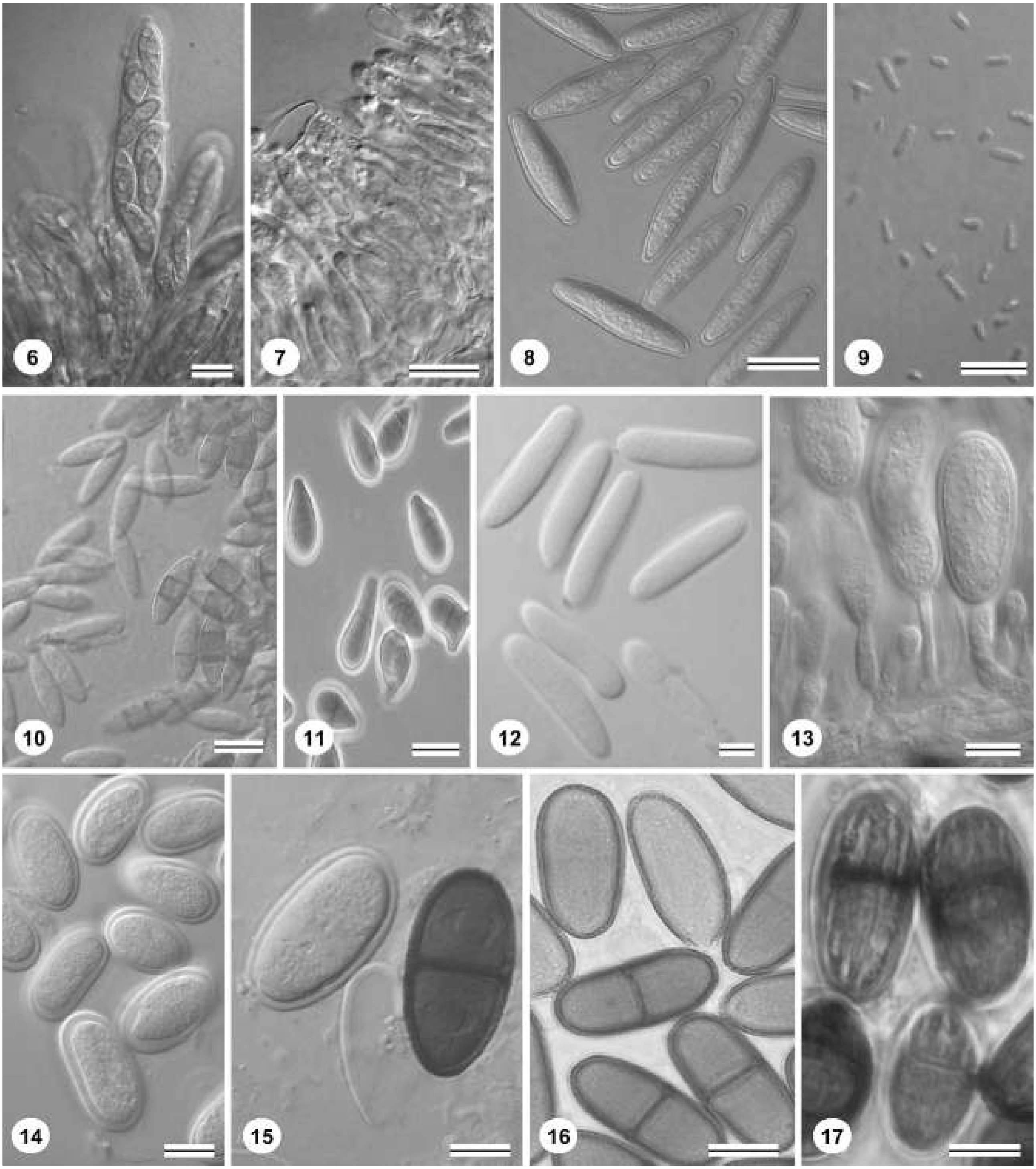
More than 20 anamorphs have been linked to *Botryosphaeria*, the most common of which include *Botryodiplodia*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia*, *Macrophoma* and *Sphaeropsis* (Denman *et al*. 2000) (Figs 1, 3, 4, 7–17). In recent years there have, however, been a number of suggestions to synonymize some of these taxa. Denman *et al*. (2000) concluded that

Fusicoccum and *Diplodia* were the only two genera that can be validated. These taxa were delineated as having typically hyaline, narrower conidia (normally $<10\ \mu m$) with thinner walls ($<0.5\ \mu m$) (*Fusicoccum*), or conidia that are wider (normally more than 10 μm) with thicker walls (0.5-2 μm) and are often pigmented when they age (*Diplodia*) (see also Zhou & Stanosz 2001; Alves *et al.* 2004) (Figs 7–17). More recently, *Dothiorella* was re-erected to accommodate the anamorphs of a group of Botryosphaeriaceae with pigmented conidia (much like *Diplodia*), which already discolored in the pycnidium, and that have teleomorphs with pigmented ascopores (= *Dothidotthia*) (Phillips *et al.* 2005) (Figs 13–17). Despite their morphological similarity to *Diplodia*, *Dothiorella* spp. are more closely related to *Fusicoccum*-like species. Barber *et al.* (2005) also showed that a number of the *Fusicoccum*-like species form a *Dichomera* synanamorph (Figs 7–12). These multi-, sometimes muriform-, septate spores are sufficiently distinct from traditional views of Botryosphaeriaceae anamorphs that they could easily be overlooked in targeted endophyte surveys that rely on morphology.

Many previous studies addressing the taxonomy and phylogeny of the Botryosphaeriaceae have suffered from an under sampling of main clades. This is either because the relationship of some groups to the Botryosphaeriaceae was not clear based on morphology, or because cultures were not available for molecular studies. A recent study included strains from most of the taxa linked to the Botryosphaeriaceae that are available in culture (Crous *et al.* 2006). This study revealed that there are at least ten lineages within the Botryosphaeriaceae (correlating well to previous anamorph lineages), most of which were subsequently described as distinct genera. A single generic name was provided for some of the new genera, accommodating the holomorph concept. Regarding lineages previously studied as endophytes, the name *Botryosphaeria* (anamorph *Fusicoccum*) does not refer to the whole group any longer, but is restricted to *B. dothidea* and closely related



Figs. 1–5 – Dissecting microscope and phase contrast compound-microscope micrographs of ascostroma and pycnidia of some Botryosphaeriaceae. 1. Singular, semi-erumpent pycnidia of a *Neofusicoccum* sp. forming on *Eucalyptus* tissue in Water Agar (WA) culture. 2. Botryose, erumpent ascomata of *Botryosphaeria dothidea* with a central ostiole. 3. Mature pycnidia of a *Neofusicoccum* sp. oozing conidia from an ostiole at the end of an extensive conical neck. 4. Singular, superficial pycnidia of *N. parvum* covered with grey mycelium, which was formed on pine needles in WA culture. 5. Median, longitudinal section through a mature ascoma of *B. dothidea*. Bar = 100 μm.



Figs. 6–17 – Light micrographs of typical sexual and asexual spores and fruiting structures of Botryosphaeriaceae. 6 Bi-tunicate asci of the Botryosphaeria-like teleomorph of *Neofusicoccum eucalypticola*. 7–9. The *Fusicoccum aesculi* asexual spores of *B. dothidea* (teleomorph). Immature conidia and conidiophores (7), narrow, fusiform and long mature conidia (8) and spermatiophores and spermatia (9). 10. Fusiform conidia of *Neofusicoccum parvum* that sometimes becomes two-septate and with a darker middle cell. 11. Dichomera-like synanamorph conidia of *Neofusicoccum* and *Fusicoccum*. 12. Bacilliform conidia of a *Pseudofusicoccum* sp. with a persistent gelatinous layer. 13–14. Immature conidia and conidiogenous cells (13) and mature conidia (14) of *Diplodia mutila*. 15. Immature hyaline and mature conidia that are pigmented and septate of a *Diplodia seriata*. (teleomorph '*B. obtusa*'). 16. Dark, septate and aseptate conidia of a *Dothiorella* sp. that often discolor while still in the pycnidium. 17. Dark, septate and striated spores of a *Lasiodiplodia* sp. Bars = 10 μm.

species. *Neofusicoccum* and *Pseudofusicoccum* were described to accommodate other Botryosphaeriaceae with *Fusicoccum*-like conidia. Botryosphaeriaceae with *Diplodia*-like anamorphs (including *Sphaeropsis*), remain accommodated in the genus *Diplodia*, but the teleomorph name, *Botryosphaeria*, is no longer available for them. Isolates of *Lasiodiplodia* grouped within the larger *Diplodia* clade in this analysis, but was not synonymized with it. More work appears necessary before this decision can be made. Most of the other lineages treated by Crous *et al.* (2006) have not been studied as endophytes.

Concepts and tools for species identification

Like in most other fungi, the morphological species concept has predominately been used to identify and describe new species of Botryosphaeriaceae. While still useful in some situations, this species concept tends to underestimate the true diversity (Taylor *et al.* 2000). This is especially true for the morphological concepts of *B. quercuum* and *B. dothidea* sensu von Arx and Müller (1954). An ecological species concept, focussed on host specialization, has been widely used to identify new taxa in *Botryosphaeria*. Host specialization is, however, not always practical, because some Botryosphaeriaceae have very wide host ranges (discussed below).

For the description of the genus *Botryosphaeria* and until the 1950's, the general morphology of the ascospores (hyaline, aseptate, shape, etc.) and stromatal and ascomatal morphology was considered taxonomically and phylogenetically informative (Figs 2, 5, 6). These characters were later shown to be inordinately variable within species and of little value for distinction between species. Since the 1960's the value of using morphological characters of the anamorphs to delimit and identify *Botryosphaeria* spp. has been realized and widely applied (reviewed in Denman *et al.* 2000). These forms are more frequently found in nature than teleomorphs and they are easily induced in culture (mostly on Water Agar (WA), or WA supplemented with sterilized pine needles or twigs of the host species) (Fig 1, 4). Conidia are also much more diverse in shape and size than the associated ascospores. Conidial characters that can be used include wall thickness, ornamentation, maturation, color, septation, shape and size (length, width, l/w and l × w) (Figs 7–17).

During the 1980's characteristics of the fungi growing in pure culture, have been commonly used to augment other characters (Gure *et al.* 2005; Pennycook & Samuels 1985; Slippers *et al.* 2004b). In general, cultures of Botryosphaeriaceae are easily distinguished from most other fungi by their grey to black, aerial mycelium and the grey to indigo-grey or -black pigment that is visible from the reverse side of Petri dishes. The appearance and color of the aerial mycelium and pigments have also aided in the delimitation and rapid identification of Botryosphaeriaceae taxa that are otherwise morphologically similar. This character is often useful for initial grouping of related isolates from a broad sampling.

In recent years, various DNA based molecular data, in particular DNA sequence data, have been used increasingly to distinguish taxa in the Botryosphaeriaceae. Differences in DNA sequences have also been successfully combined with morphological characteristics to identify and describe Botryosphaeriaceae taxa (for example Denman *et al.* 2003; Phillips *et al.* 2005; Smith *et al.* 2001). In some cases this approach has revealed surprising diversity. For example, *B. dothidea*, which was the first reported endophyte of the group occurring in *Eucalyptus*, is now known to be rare on this host. On *Eucalyptus*, this name could refer to any one of ten species (Burgess *et al.* 2005; Mohali *et al.* 2007; Slippers *et al.* 2004c).

Most taxonomic studies on Botryosphaeriaceae using DNA sequence differences have used ITS rDNA phylogenies, but this single gene can underestimate the true species diversity among closely related or cryptic species. In this regard, multiple gene sequence concordance phylogenies have been successfully applied to identify cryptic species if the Botryosphaeriaceae, previously overlooked or of uncertain identity (Burgess *et al.* 2005; de Wet *et al.* 2003; Phillips *et al.* 2005; Slippers *et al.* 2004a,b,c). Most commonly data from Translation Elongation Factor 1- alpha (EF1-α) have been combined with ITS sequences.

Simple Sequence Repeat (SSR) markers can be used very powerfully to indicate species boundaries for cryptic species in the Botryosphaeriaceae. This can be seen in cotemporary studies of the important pine pathogen *Diplodia pinea* which has been studied extensively for many years. RAPD markers, combined with morphology and epidemiology, have characterized four 'morphotypes' known as the A, B, C and I morphotypes of this taxon (de Wet *et al.* 2000; Hausner *et al.* 1999; Palmer *et al.* 1987; Smith & Stanosz 1995). There is, however, variation in the characters used to distinguish these morphotypes and single gene phylogenies do not separate all morphotypes either. Microsatellite markers have revealed that there is no genetic exchange between the B morphotype and the A and C morphotypes, and that the 'I' morphotype represents *B. obtusa* (Burgess *et al.* 2001b). Subsequently, comparison of sequence data for some of these markers, as well as for some genic coding regions, has confirmed that the B morphotype is a distinct species, *D. scrobiculata* (de Wet *et al.* 2003). Similar tools have also been applied to *N. parvum* and *N. ribis* population and species distinctions (Slippers 2003).

After the identification of a species using the methods described above, there is usually a need for a rapid and effective tool to identify the species. This is especially true where a specific host (especially cultivated and introduced hosts) is infected by a complex of Botryosphaeriaceae species (Jacobs 2002; Mohali *et al.* 2007; Slippers *et al.* 2004c, 2007). In this regard species-specific primers and PCR RFLP profiles have proven useful and effective identification tools.

Species-specific primers have been applied to identify subspecies or species groups in *Botryosphaeria* (see below under 'Isolation and detection *in vivo*'). This tool has also been applied widely for the identification of other fungi, and could become a powerful aid used to study infection levels and patterns of Botryosphaeriaceae species and communities. Various factors, however, hamper the development of species-specific primers for some Botryosphaeriaceae. The ITS rDNA region has been shown to be insufficient to distinguish closely related species (de Wet *et al.* 2003; Slippers *et al.* 2004a). Even where ITS sequences are sufficient to distinguish closely related species, such as *N. eucalyptorum* and *N. eucalypticola,* and *N. luteum* and *N. australe*, the polymorphisms are spread across the fragment (Slippers *et al.* 2004a,c). Primers will, therefore, differ only by one base pair, which might not be sufficient or robust under all conditions. Species-specific primers between these closely related species will need to be developed for other regions of the genome. For example, the EF1-α region is consistently more variable than the ITS rDNA region. Unfortunately, polymorphic sequences between the some closely related species, e.g. *N. ribis* and *N. parvum*, and *D. pinea* and *D. scrobiculata*, are not close enough in any part of the regions sequenced thus far, to allow the development of robust primers.

Like species-specific primers, PCR Restriction Fragment Length Polymorphism (RFLP) fingerprints can provide effective tools to rapidly and reliably identify larger numbers of isolates that would be impractical to identify otherwise. RFLP profiles of the ITS rDNA region (Jacobs 2002; Mohali *et al.* 2007; Slippers *et al.* 2004c, 2007), a larger portion of the rDNA region (Alves *et al.* 2005) or some unidentified SSR containing regions (Slippers 2003) has been used to distinguish among distant and closely related species of the Botryosphaeriaceae. This technique overcomes the need for a continuous group of polymorphic bases, because restriction enzymes (RE) recognize single nucleotide polymorphisms (SNP). Unfortunately no single gene region is sufficient to distinguish all species, because not all SNP's represent restriction sites, especially between some closely related species.

4. Isolation and detection *in vivo*

In a typical sampling effort, branches and leaves are collected, transported to the laboratory, stored at 4 °C and then processed as soon as possible. In cases where endophytism is not strictly implied, samples are left for a week or more, and then processed. Presumably during this period, small infections might colonize more tissue, making the endophyte easier to isolate. A danger with longer incubation times is, however, that epiphytic populations of fungi might penetrate the tissue and give a skewed view of the real endophytic community. To overcome this problem, surface disinfestation should precede incubation.

Typical isolation procedures for Botryosphaeriaceae have followed widely used surface disinfestation by a succession of washing steps in EtOH and household bleach (Fisher *et al.* 1993; Pavlic *et al.* 2004; Smith *et al.* 1996a). Branches and leaves are often first washed in running tap water. Branches or twigs can then be debarked and isolated directly from exposed tissue. Alternatively, and desirably, twig samples, as well as leaves should be surface disinfestated. Typically this is done by sequential washing in 70 % EtOH (for 30 s or more), household bleach (NaOCl; 3.5-5 % available chlorine) (between 1-5 min.), and >95 % EtOH (30 s or more), before finally rinsing in sterile water (once or twice). To ensure that all epiphytes are killed when the endophyte community is to be characterized, disinfected samples can be pressed on separate media and a sample of the final sterile rinse water can be plated as controls.

Most Botryosphaeriaceae grow relatively fast and compete strongly for resources with other fungi on general media. Half strength (2 %) Malt Extract Agar or commercial Patato Dextrose Agar have thus been commonly used to isolate them. These media are sometimes supplemented with antibiotics (e.g. chloramphenicol) to suppress bacterial growth. On such primary isolation plates, the Botryosphaeriaceae typically have mycelium that is pigmented, a greenish brown or grayish color and that become dark gray or grayish-blue to black with time. Selective media have been developed to isolate *D. pinea* and *L. theobromae* from *Pinus* (Blodgett *et al.* 2003; Cilliers *et al.* 1994; Swart *et al.* 1987). These media could have wider application in isolating Botryosphaeriaceae from other hosts.

Increasingly effective PCR-based molecular and phylogenetic tools have been developed for *in vivo* detection of DNA of specific species or representing communities of organisms (as examples, see Higgins *et al.* 2007 and Martin & Rygiewicz 2005 for fungi in general, and Moon *et al.* 1999 for specific groups of grass endophytes). These tools represent an exciting opportunity to characterize general endophytic communities in woody plants, and in particular also the Botryosphaeriaceae.

Species or group (closely related species) -specific primers for standard PCR reactions have been developed for *D. pinea*, *D. scrobiculata*, *B. obtusa* and a *Botryosphaeria* sp. (Flowers *et al.* 2003; Ma & Michailides 2002; Smith & Stanosz 2006). Studies applying these techniques could detect levels of fungal DNA as low as 0.93 pg in bud samples to 10-100 pg in bark samples. There is now scope to develop these techniques for these and other species to be used to quantify endophytic infections. For example, Luchi *et al.* (2005a) used species specific primers in a real-time PCR approach to detect and quantify the level of Botryosphaeriaceae *in vivo*, albeit after artificial infection and not natural endophytic infections.

One concern with direct detection tools is that the presence of epiphytic fungi can lead to false positive results (see Santamaria & Bayman 2005). Tools are needed to overcome this shortcoming, either by removing covering layers of plant tissue (i.e. bark or epidermis) and thereby removing epiphytic fungal propagules. Another approach would be to wash off or disrupt epiphytic fungal propagules and their DNA (starting by testing the efficiency of current isolation methods for this purpose).

Important variables that have not always been recorded during collections and isolations of Botryosphaeriaceae are the age of the leaves, time of collection (to consider seasonal or other temporal variations) and the size of the isolation units. It has been shown that the level of endophyte infection increases with the aging of the leaves (Arnold & Herre 2003; Saikkonen 2007; Sieber 2007, personal observations on *Eucalyptus*). The size of leaf or twig pieces can also significantly affect the levels and diversity of species recovered (Arnold *et al.* 2001; Petrini *et al.* 1992; Smith 2001). Furthermore, collections during different seasons have been shown to influence endophyte communities (see Saikkonen *et al.* 1998; Saikkonen 2007 for reviews). It is also clear from numerous studies that there is geographical or spatial structure in endophyte communities (Arnold *et al.* 2001, 2003; Carroll & Carroll 1978; Fisher *et al.* 1993; Higgins *et al.* 2007; Saikkonen *et al.* 1998; Saikkonen 2007, and discussed elsewhere in the paper for Botryosphaeriaceae). It is important to consider the above mentioned variables when collecting Botryosphaeriaceae endophytes, especially if direct comparisons of fungal species or communities are to be made between sites, hosts or time points.

5. Occurrence and diversity of endophytic Botryosphaeriaceae in woody plants

The majority of the genera in the Botryosphaeriaceae (Crous *et al.* 2006), include members that have been described as endophytes. These include *Guignardia*, *Botryosphaeria* (anamorph *Fusicoccum*), *Dothidotthia* (anamorph *Dothiorella*), *Neofusicoccum*, *Pseudofusicoccum*, *Lasiodiplodia* and *Diplodia*. Some of these endophytes are very common, dominating endophyte communities in *Eucalyptus* and *Pinus* in some environments (Burgess *et al.* 2006; Smith *et al.* 1996a,b). A specific host can also be infected by a diverse community of endophytes, as has emerged in a recent South African study where eight species were isolated as endophytes of *Syzygium cordatum*, often co-occurring on the same plant (Pavlic *et al.* 2007). From this information, and personal observations that Botryosphaeriaceae are present in most woody plants with which we have worked, it appears that endophytism is common to most species of the family.

The Botryosphaeriaceae have been described from virtually all woody hosts examined, including many gymnosperms and angiosperms (but not all these were as endophytes). In reviewing the literature of recently (since the early 1990's when their endophytic nature became better established) described Botryosphaeriaceae, it is clear that these fungi are still predominantly described for their roles as pathogens. This is where they become most obvious, and for economic reasons, where most workers focus their efforts. It is, however, disappointing to see that in many of these situations, little effort is made to isolate these fungi from asymptomatic tissue, despite the critical role that such infections would play in the epidemiology of the diseases.

In agricultural and forestry plant communities that have been examined, woody plants have often displayed high levels of endophytic Botryosphaeriaceae colonization (Smith *et al.* 1996a,b; Burgess *et al.* 2006). In the few situations examined, infection levels of native plant communities have reflected lower levels of endophytic Botryosphaeriaceae infection. For example, *B. dothidea* (possibly incorrectly identified; see Slippers *et al.* 2004a) was uncommon in native *Eucalyptus* in Australia, but common in non-native *Eucalyptus* in England and South Africa (Fisher *et al.* 1993; Smith *et al.* 1996a,b). *Neofusicccum australe* was isolated from 79 % of twigs of *E. globulus* plantation trees in western Australia (planted outside their native range), but in native ranges in eastern Australia and Tasmania two other species, *N. eucalyptorum* and *N. eucalypticola*, predominated from only 17 % of the isolations (Burgess *et al.* 2006). From these, and other personal observations, it would appear that the Botryosphaeriaceae are particularly successful as opportunistic endophytic colonists. This is especially true in hosts in disturbed or non-optimal environments, when plants are under stress, or where the endophytic niche is left 'open' (i.e. when a host grows in a non-native environment where its normal sweet of horizontally acquired endophytes are not present).

6. Host association

Despite the fact that members of the Botryosphaeriaceae commonly infect gymnosperms, they are most common and diverse on angiosperms. Of the 2000 species (considering the names contained in *Botryosphaeria*, *Fusicoccum*, *Dothiorella*, *Diplodia* and *Lasiodiplodia* in Index Fungorum; http://www.indexfungorum.org), fewer than 50 appear to have been described from gymnosperms. A phylogenetic analysis of the available sequence data in GenBank indicated that the most common ancestor of the extent Botryosphaeriaceae most likely lived on angiosperms (Juanita de Wet and co-workers, manuscript in preparation). This might simply reflect angiosperm vs. gymnosperm diversity if strict co-evolution is expected, but many Botryosphaeriaceae are not strongly host specific and their diversity would consequently be less affected by the host diversity.

In terms of host association of the Botryosphaeriaceae, two views have been prevalent in the past. Many taxonomists have considered the ability to infect a specific suite of hosts as delimiting species. Many teleomorph and anamorph species in this family have thus been described based on host. Examples include *B. quercuum* (from *Quercus*), *B. ribis* (from *Ribes*), *B. mali* (from *Malus*), *B. vitis* (from *Vitis*), and many other names. On the other hand, some authors have presumed less host specialization. This view has contributed to a very broad, 'super-species' concept for some *Botryosphaeriaceae* such as *B. ribis*, *B. obtusa*, *B. dothidea*, *B. quercuum* (sensu von Arx & Müller 1954) and other species.

Certain well-studied species have in recent years been confirmed to infect a wide range of hosts ('host neutral') using molecular tools, e.g. *B. dothidea*, *D. seriata* (= '*B.*' *obtusa*) *N. parvum* (= *B. parva*), *N. australe* (= *B. australis*), *L. theobromae* (Burgess *et al.* 2005, 2006; Pavlic *et al.* 2007; Slippers *et al.* 2004a,b, 2007). In contrast, some well characterized species are clearly specialized on certain host genera or specific plant families in a defined area, e.g. *D. pinea* and *D. scrobiculata* on *Pinus* and occasionally other conifers, *N. eucalyptorum* and *N. eucalypticola* on *Eucalyptus*, *Saccharata protea* and *N. proteaarum* on Proteaceae (Burgess *et al.* 2004a,b; Denman *et al.* 2003; Slippers *et al.* 2004c; Smith *et al.* 2001; Swart *et al.* 2000). In these examples, and in a number of ongoing unpublished studies with which we are involved, closer examination of Botryosphaeriaceae from indigenous hosts in natural ecosystems often reveal these distinct, and more specialized, species. They have commonly been overlooked in the past, because of morphological similarities or uncertainty regarding the phylogenetic value of small morphological differences, or because native, non-commercial trees are under sampled. Many of these species also occur sympatrically on the same hosts.

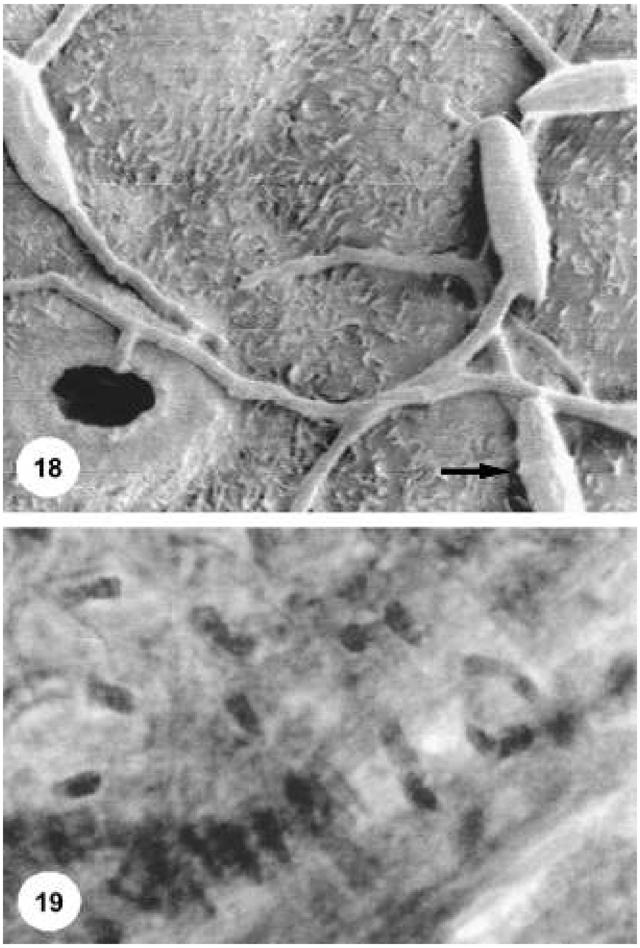
Host species specificity of endophytic Botryosphaeriaceae is implied in the findings of Smith *et al.* (1996a), who showed that *D. pinea* infects different *Pinus* species to different levels in the same environment (*P. radiata* 90 %, *P. patula* 50 %, *P. taeda* 10 % and *P. elliottii* 0 %), correlating to their known resistance to *D. pinea* die-back (Swart & Wingfield 1991). Similarly, Botryosphaeriaceae infection levels vary in *Eucalyptus* spp. in South Africa (*E. smithii* 93 %, *E. camaldulensis* 77 %, *E. grandis* 63 % and *E. nitens* 57 %) (Smith *et al.* 1996a). Luchi *et al.* (2005b) also showed species specific differences in recognition of *P. nigra* by *D. pinea* and *D. scrobiculata* (see below under 'Infection and colonization'). This level of host specificity has, however, not been dissected thoroughly in the Botryosphaeriaceae. This information can be potentially important to understand the risk posed by these fungi, and to devise control strategies for them, as well as to understand the mechanisms governing host specificity or neutrality.

When considering potential ‘host neutral’ species, or species with very wide hosts ranges, their host affinity seem strongly influenced by the environment (some examples discussed elsewhere in the review; see also Saikkonen 2007 for other endophytes). For example, *N. australe* is very rarely found on *Eucalyptus* in eastern Australia (only found once from numerous isolations (Burgess *et al.* 2006; Slippers *et al.* 2004c)), despite being common on native *Acacia* spp. and other hosts in the area (Slippers *et al.* 2004b). In contrast, in western Australia, it is the dominant species infecting *Eucalyptus*, as well as 11 other native and non-native hosts (Burgess *et al.* 2006). In South Africa, this fungus does not occur commonly on *Eucalyptus*, yet it is relatively common in the area on native *Syzygium* and introduced *Vitis*, and to a lesser extent native *Widdringtonia* and introduced fruit trees (Pavlic *et al.* 2007; Slippers *et al.* 2005c, 2007). These site-specific factors affecting host affinity might include abiotic environmental factors, Botryosphaeriaceae inoculum pressure originating from native or established exotic hosts, competition from different endophyte communities, physiological or physical variation in the host in different regions or some other factors yet to be identified (see Saikkonen 2007 for a discussion of such forces). Whatever these factors are, they clearly play a very significant role in defining the Botryosphaeriaceae community in a particular area, often more so than the host, and studies to answer these questions are likely to be valuable.

7. Infection and colonization

In the past, the general dogma was that infection by Botryosphaeriaceae occurred *via* wounds (von Arx & Müller 1954; Smith *et al.* 1994). This is obvious from cankers that begin to develop from wounds on leaves, branches or stems (see ‘Disease expression’ below). A number of studies have, however, shown that they these fungi can infect directly through lenticels, stomata or other openings on healthy plants (Brown & Hendrix 1981; Kim *et al.* 1999; Michailides 1991; Smith 2001) (Figs 18, 19). This leads to endophytic infections, unless the host is under stress (see ‘Disease expression’ below). Virtually all plant parts, from the bark and xylem of stems, branches, leaves, flowers, fruit, seed capsules and cones and seeds, have been reported as host to latent Botryosphaeriaceae (Cilliers *et al.* 1995; Johnson *et al.* 1992; Kim *et al.* 2001, 2004; Lupo *et al.* 2001; Smith *et al.* 1996a,b).

Botryosphaeriaceae have been characterized as seed-borne or infecting seed. There is, however, little evidence that these infections of seeds result in systemic infections in the plants as they develop. Seeds of *Pinus* have been shown to have latent infections of *L. theobromae* and *D. pinea* (Cilliers *et al.* 1994, 1995; Smith *et al.* 1996a). These internal seed infections have been implicated in the movement of these pathogens around the world (Burgess *et al.* 2001a, 2004b; Smith *et al.* 2000). A recent study of healthy seeds of native *Prunus* and *Podocarpus* trees in Ethiopia, revealed four species belonging to three genera of the Botryosphaeriaceae, of which three species were undescribed (Gure *et al.* 2005). This aspect of the biology of Botryosphaeriaceae deserves more intensive study in the future, especially to determine how seed-borne infections might infect growing plants. Knowledge of this stage of the endophytic life cycle is critical in order to understand the influence of seed movement on spread of the Botryosphaeriaceae, as well as their ecological role as endophytes.



Figs. 18–19 – Scanning electron micrograph of germinating conidia of a *Neofusicoccum* sp. entering stomata of a *Eucalyptus* leave. Localized endophytic infections in discolored leave tissue of a *Eucalyptus* leave after infection by a *Neofusicoccum* sp. (Reproduced from Smith 2001).

Endophytic infections of Botryosphaeriaceae of fruits are common. These infections often lead to soft brown rot of fruit shortly before or after harvest, resulting in extensive losses (Johnson *et al.* 1992; Kim *et al.* 2001, 2004). Like other plant parts, fruits are infected *via* natural openings, but appresoria on the fruit surface have also been observed (Kim *et al.* 1999). Infection of the bud ends of fruit can also occur through the pedicle (Johnson *et al.* 1992). Fruits can be infected early on in their development, but disease symptoms typically appear as sugar levels rise during ripening, or during storage (Johnson *et al.* 1992; Parker & Sutton 1993).

A series of detailed studies on the infection of *B. dothidea* on apple fruits (Kim *et al.* 1999, 2001, 2004) have described aspects of the biochemistry of Botryosphaeriaceae infections that might apply to systems beyond those of fruit infections. These studies showed that microbodies and lipid globules develop in *B. dothidea* hyphae during infection, which were functionally defined as glyoxysomes. Their findings suggest that the glyoxysomes enable the fungus to endure the nutrient deficiency and host defense responses during latent periods and adverse conditions.

Luchi *et al.* (2005b) studied the response of *P. nigra* (native to Europe) to infection by *D. pinea* (common and most likely native to Europe) and *D. scrobiculata* (common and most likely native to some parts of North America). Traumatic resin ducts (TRD) quickly (after 4 d) formed when inoculated with *D. scrobiculata*, and no tissue colonization followed. TRD formed slower in control wounded trees (after 12 d). TRD's did not form in response to *D. pinea* infection, and extensive tissue colonization followed. This suggests a specific host-fungal recognition and following interaction. It also correlates with the fact that *D. scrobiculata* has not been found commonly infect *Pinus* outside the USA, despite apparently having ample opportunity to spread (Burgess *et al.* 2004a,b).

Endophytic Botryosphaeriaceae infections of woody hosts are thought to predominantly occur through horizontal transmission, i.e. individual infections *via* spores. Smith (2001) show that the individual infections in a leaf represent up to 14 different vegetative compatibility groups (clonal entities), and clearly arose from multiple infections (Figs 19, 20). Nursery plants typically have lower levels of Botryosphaeriaceae infection unless exposed to the proximity of mature trees which provide a source of inoculum (Palmer *et al.* 1988; Stanosz *et al.* 2005). This is the most common form of transmission amongst other endophytes of woody plants, unlike grass endophytes, which are often vertically transmitted from mother plants to offspring, *via* seed or vegetative propagules (Arnold & Herre 2003; Carroll 1988; Clay & Schardl 2002; Saikkonen *et al.* 1998; Sieber 2007; Stone & Petrini 1997). This is an important difference in understanding the interaction between the plant and fungus (see Saikkonen *et al.* 1998; Saikkonen 2007). In mutualistic symbioses, more consistent interactions through vertical transmission, usually also enforce asexual reproduction and a reduced diversity on the mutualistic partner, which is thought to stabilize the interaction (Herre *et al.* 1999). In fact, horizontal transmission and multiple symbiont genotypes are thought to contribute to the unraveling of mutualisms. Following this argument, the interactions between woody plants and Botryosphaeriaceae appear to be more casual, unlike obligate mutualists (Saikkonen *et al.* 1998; Saikkonen 2007; Sieber 2007). Carroll (1988) defined these types of interactions between plants and fungi as 'inducible mutualists'.

Carroll (1988) noticed that many endophytes of woody plants produce slimy spore masses that are associated with rain dispersal. This is also true of the Botryosphaeriaceae where both the teleomorphs and anamorphs generally produce a slimy mass of spores from a central ostiole in the pycnidium or perithecium (Figs 1, 3). While this is not an adaptation specific to Botryosphaeriaceae or fungal endophytes of trees alone, it could have some advantages for these fungi (Carroll 1988). It may help adhesion to host surfaces during wet periods and most likely more local dispersal onto a specific host and its surrounding offspring onto plants which is more likely to share genetic affinities.

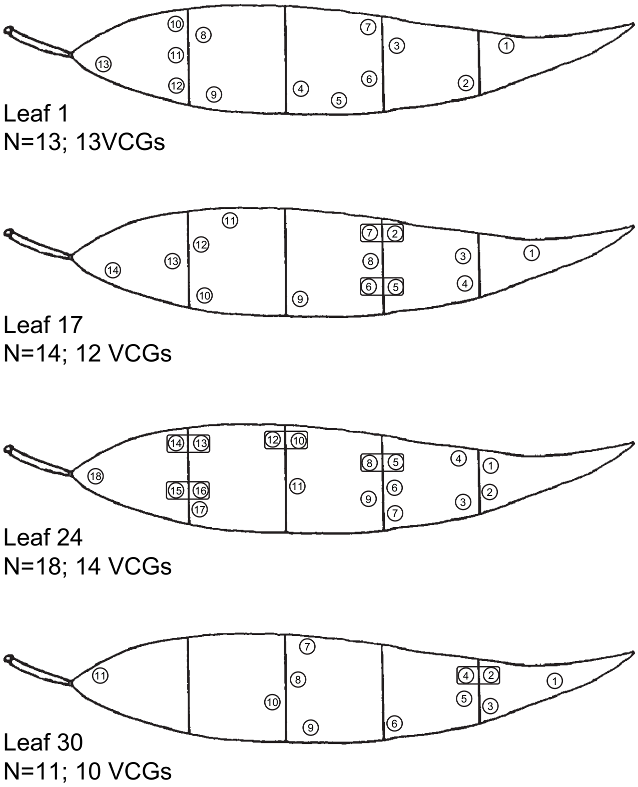


Fig. 20 – A redrawn selection of sectioned, surface disinfested *Eucalyptus* leave images, showing origin of isolates of *Neofusicoccum* spp. Isolates were paired in all possible combinations on artificial medium to determine vegetative compatibility groups (VCGs) (identical genotypes). Up to 14 VCG's were recovered per leave, clearly originating from individual infections and showing little lateral colonization of the leave after infection. Identical VCGs were always opposite section cuts. (Redrawn from Smith 2001).

Dispersal and endophytic infection by Botryosphaeriaceae can occur *via* ascospores or conidia. Interestingly, population genetic studies have shown that *D. pinea* is probably obligately asexual (Burgess *et al.* 2004b) and spore trapping has shown that this fungus spread species exclusively *via* conidia (Swart & Wingfield 1991). These observations correlate well with limited diversity observed in this species over large areas (Burgess *et al.* 2004b). Some species in which a sexual state is known, also display clonality or identical molecular genotypes over large areas (e.g. *N. parvum* in Slippers (2003)); *B. dothidea* in Michailides (1991) and Ma *et al.* (2004); and *L. theobromae* in Burgess *et al.* (2005). This indicates that either conidia or homothallic ascopores are an important, and often dominant, form of dispersal and infection in these fungi. On the other hand, populations of *D. scrobiculata*, for which no sexual stage has been found, reveal signs of sexual recombination in multilocus DNA analyses (Burgess *et al.* 2004a). Similar analyses of *N. parvum* populations from *Eucalyptus* confirmed this view that, despite widespread asexual reproduction, sexual outcrossing does occur, albeit less frequently (Slippers 2003). These observations correlate with observations that anamorph fruiting structures are much more common in nature, and culture, than teleomorph structures for many Botryosphaeriaceae. The extent of outcrossing, and the way in which it influences population structure of endophytic Botryosphaeriaceae has not been considered for most other species, but results of such studies are likely to positively influence our understanding on endophytism in the Botryosphaeriaceae (Saikkonen *et al.* 1998).

8. Disease expression

Disease expression for species of Botryosphaeriaceae is almost exclusively associated with some form of stress or non-optimal growth conditions of trees (Blodgett & Stanosz 1995; Ma *et al.* 2001; Paoletti *et al.* 2001; Schoeneweiss 1981; Smith *et al.* 1994; Stanosz *et al.* 2001, an numerous others). Stress conditions that have been linked to the Botryosphaeriaceae in these studies include drought stress (most commonly), extensive physical damage (e.g. hail), biological stresses such as damage by other pathogens or insects, frost or heavy snow, interplant competition resulting from overstocking, or planting species or varieties on unsuitable sites (elevation, soil type, temperature, etc.). Various diseases symptoms have been linked to members of the Botryosphaeriaceae, including twig, branch and main stem cankers; die-back of leaders, shoots or whole branches; seed capsule abortion; collar rot, damping off or blight of seedlings; root cankers; blue-stain; decline; and in severe cases death of whole trees (Ahumada 2003; Bega *et al.* 1978; Brown & Britton 1986; Gure *et al.* 2005; Johnson *et al.* 1992; Lupo *et al.* 2001; Michailides 1991; Sánchez *et al.* 2003; Smith *et al.* 1994; Swart & Wingfield 1991) (Figs 21–33).

The impact of diseases caused by species of Botryosphaeriaceae, especially in forest situations, is difficult to judge. Nevertheless, where enumerations have been attempted, damage have been significant. In South African pine plantations, the damage due to *D. pinea* has been calculated to between 11 % and 28 % volume loss, and up to 55 % potential loss of production after hail damage and die-back (Zwolinski *et al.* 1990). It is also one of the main factors precluding planting of *P. radiata*, desirable for its wood properties, in the majority of the pine-growing area of South Africa (Swart & Wingfield 1991). *Diplodia pinea* is currently considered the most economically important pathogen of pine forestry in South Africa, New Zealand and other parts of the Southern Hemisphere (Reay *et al.* 2006; de Wet *et al.* 2000; Smith *et al.* 2001). In the USA it has also caused large scale losses of seedlings and trees under stress (Blodgett & Stanosz 1995; Stanosz *et al.* 2001), and in Europe it is currently responsible for widespread die-back of *Pinus nigra* (Luchi *et al.* 2005a,b; Maresi *et al.* 2002).

An intriguing observation regarding Botryosphaeriaceae as pathogens is that the most damaging species are those that have very wide host and/or geographic ranges, such as *L. theobromae* (vs. *L. gonubiensis*), *N. parvum* and *N. australe* (vs. *N. eucalyptorum* and *N. eucalypticola*), *D. pinea* (vs. *D. scrobiculata*), *B. dothidea*, *B. obtusa*, and others. In artificial inoculations, these wide host and geographic range species are frequently the most pathogenic (Ahumada 2003; de Wet *et al.* 2000; Pavlic *et al.* in press; van Niekerk *et al.* 2004; Slippers, unpublished data (*Neofusicoccum* spp. on *Eucalyptus*)). They are also frequently the dominant fungi to emerge when isolations are made from diseased tissue. Possible explanations might be that the ability of species of Botryosphaeriaceae to infect a wider range of plants, frequently brings them into contact with hosts that have not co-evolved resistance to them (Parker & Gilbert 2004; Slippers *et al.* 2005b). Furthermore, species with broader host ranges are likely to become established more easily in new areas, as their establishment will not depend on the presence of specific hosts.

Not all species with broad host ranges cause diseases on all the hosts they infect, in all areas. For example, *B. dothidea* is a very serious pathogen of fruit and nut trees in the USA, but it seems to be absent or rare on these hosts in South Africa and other regions (Michailides 1991; Pavlic *et al.* in press; Slippers *et al.* 2007). This is possibly an effect of local environmental pressure, or competition from native Botryosphaeriaceae or other endophytes for the same niche, as discussed elsewhere.

9. Quarantine, spread and climate change

In general, quarantine systems are not well suited to consider latent pathogens which live endophytically in healthy plant material for an extended period of time. The Botryosphaeriaceae provide an excellent example of this fact. Large quantities of seed and especially plant material are likely to contain asymptomatic infections of these fungi. As such, and also because they are often not considered primary pathogens of quarantine concern, they would easily be overlooked. Even where a pathogen such as *B. dothidea* appears on a quarantine list, it would be difficult to imagine that quarantine procedures could be effectively applied against its endophytic infections. Once some species in the Botryosphaeriaceae has been introduced into a new environment, they would likely have the capacity to jump to new hosts that might have no co-evolved resistance mechanisms against it, potentially causing serious damage (Parker & Gilbert 2004; Slippers *et al.* 2005b).

A number of closely studied examples are revealing the extent to which human mediated movement of the Botryosphaeriaceae has occurred around the world. *Diplodia pinea*, a pine-specific species, provides and excellent example to illustrate this point. *Pinus* spp. are non-native in Australasia and South Africa, but they have been established extensively in plantations. *Diplodia pinea*, however, is a very common endophyte in tissues of these trees and it has clearly been introduced from the native ranges of *Pinus* (Burgess *et al.* 2001a; Smith *et al.* 1996a, 2000). The genetic diversity of the fungus in some countries, such as South Africa, is very high; higher than some studied native populations (Burgess *et al.* 2001a, 2004b; Smith *et al.* 2000) (Fig 34). As *D. pinea* is an asexual fungus (see discussion above), this diversity in introduced environments is thought to be the result of multiple introductions from more than one area of the native range of *D. pinea* (Burgess *et al.* 2001a, 2004b; Smith *et al.* 2000). Shared vegetative compatibility (VC) groups and multilocus genotypes between Australia, Tasmania and New Zealand, further reflect the high level of anthropogenic movement of this fungus (Burgess *et al.* 2001a, 2004b). Other examples include *N. eucalyptorum* and *N. eucalypticola*, endophytes that are seemingly specific to *Eucalyptus* and native to eastern Australia, and which have apparently been introduced with the host into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South Africa and Chile (Ahumada 2003; Burgess *et al.* 2006; Slippers *et al.* 2004c). There are many more examples of this trend and these will continue to become more obvious as DNA-based tools are applied to study them.

The lack of host specificity in some Botryosphaeriaceae means that they can cross-infect native and introduced hosts, moving in both directions causing endophytic infections and disease (Parker & Gilbert 2004; Slippers *et al.* 2005b). Slippers (2003) showed that *N. parvum* shares multilocus simple sequence repeat (SSR) genotypes between isolates from native and introduced hosts in Australia (*Eucalyptus* and *Tibouchina*) and New Zealand (*Araucaria*, *Populus* and *Tibouchina*), and high similarity between genotypes from these regions and from *Eucalyptus* in South Africa. Mohali *et al.* (2005) used markers to show that there was no barrier to gene flow between populations of *L. theobromae* occurring endophytically in introduced *Pinus*, *Eucalyptus* and *Acacia*. Pavlic *et al.* (2007) found eight species of the Botryosphaeriaceae infecting native *Syzygium* in eastern areas of South Africa. One of the most common and most pathogenic species, *N. parvum*, also commonly occurs on *Eucalyptus* and *Mangifera indica* (Mango) in the same areas (Jacobs 2002; Slippers *et al.* 2005a). Other Botryosphaeriaceae from *Syzygium* are also known from different hosts, e.g. *N. mangifera* (from mango in Australia), *N. australe* and *L. theobromae* (both from various other hosts, including *Eucalyptus*, in other regions of the world).

Botryosphaeriaceae are difficult to control once they have been introduced into a new area (Swart & Wingfield 1991). They are likely to infect all the plants of a given host lineage (host range depending on the species). Chemical control of these infections is extremely difficult on a large scale (forest or plantation), if not impossible, and environmental hazards would also not validate such treatment. Removal or treatment of diseased parts of trees is possible in intensively managed orchards (Brown-Rytlewski *et al.* 2000; Flowers *et al.* 2001). This, together with sanitation to reduce spore loads, can help to reduce disease (Brown-Rytlewski & McManus 2000; Michailides 1991; Palmer *et al.* 1988; Stanosz *et al.* 2005). But over time, new symptoms are likely to continue appearing from other endophytic infections, so the control is not absolute either. In addition, susceptible species can be replaced with resistant species (Swart & Wingfield 1991), but this might not always fit market requirements. Breeding for resistance might be possible, but resistance is likely to be easily overcome by high geneflow and sexual reproduction. The best approach to control is, therefore, prevention of entry of pathogenic species and genotypes.

Predictions from climate change models list consequences that could have a full range, positive, neutral and negative, effects of host-pathogen/pest interaction (Coakley *et al.* 1999). Very little research has been undertaken on this topic for endophytes and particularly the Botryosphaeriaceae. There are at least two major, mostly negative, ways in which climate change can affect endophyte (in particular Botryosphaeriaceae)-plant interactions:

- Climate change could add additional stress or pressure on woody plants in forest and agricultural situations through extreme weather conditions (e.g. either heat, cold, drought or extreme wetness) or expansion of known pest and disease host ranges. Botryosphaeriaceae, like many other endophytes, cause diseases when their hosts are under stress. Under 'normal' conditions this might favor the host, as discussed later ('Ecology and Evolution'). Yet, when such stress is ubiquitous over a large area, and affecting much of a hosts distribution, the resulting diseases from the otherwise mild diseases caused by endophytes, might be severe (Desprez-Loustau *et al.* 2006).
- Changes in the environment might limit the ranges of certain endophytes, including certain Botryosphaeriaceae, and thus change the endophyte community infecting a particular host in a specific area. If these endophytes played a protective role (as postulated by Carroll (1988); Arnold *et al.* (2003)), then these woody hosts would be left potentially vulnerable in their absence. This would be more severe in cases where the host-endophyte interaction was specific, compared to generalist species. A new suite of endophytes is likely to infect these hosts, and Botryosphaeriaceae seem particularly effective in this regard (see discussion under host association). Whether these "new" endophytes would be as effective as potential mutualists, or become increasingly antagonistic, remains to be revealed.

10. Ecology and evolution

It is clear that endophytes are ubiquitous in all woody plants, and at least as diverse and abundant as ectomychorrhizae (Arnold *et al.* 2000; Higgins *et al.* 2007; Sieber 2007). Given this abundance, it is hard to believe that they do not have a major effect on plant communities (Carroll 1988). Clay (2001) argues that these microbial symbionts are important determinants of plant community structure and likewise of the insect communities that live on these plants. In many grass communities,

Figs. 21–33 – Typical disease symptoms associated with Botryosphaeriaceae, showing they extent of damage they can cause in different hosts. 21–24. Cankering, die-back and death of leaders and side branches of *Eucalyptus*, with bluish-black discoloration of the pith material in a young stem, typical of *Neofusicocum* and *Fusicoccum* damage on *Eucalyptus*. 25–26. Infected side branches or wounds can also affect the main stem, with callus over an old canker wound. 27–28. *Lasiodiplodia theobromae* causes die-back and wood discoloration in various parts of the tropics, here on *Acacia mangium* in Indonesia. 29–33. *Diplodia pinea* causes severe damage to *Pinus* spp. around the world. Infection, cankering and die-back is often associated with hail (29) or other wounds (30). Infections can also occur from endophytic infections in the cone, *via* the connecting branchlet and into the main branch (31). Die-back from endophytic infections without apparent wounds, but where plants are under stress (32). Pine species are not all equally susceptible, with *Pinus patula* (33 left) showing severe die-back after hail, while *P. greggii* (33 right) is unaffected.

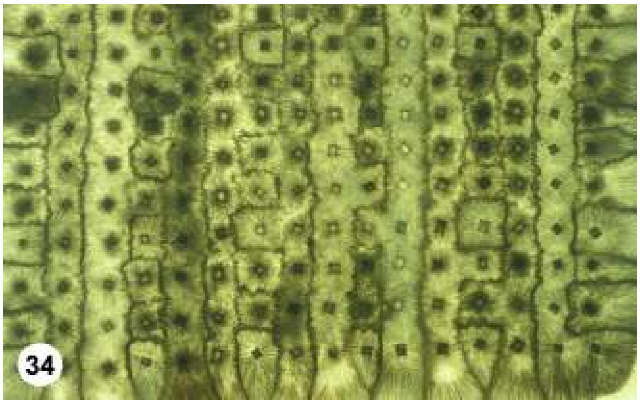

Fig. 34 – Vegetative compatibility group amongst *Diplodia pinea* isolates from Pines in South Africa. Confluent isolates indicate identical genotypes, while distinct genotypes are delineated with a dark interaction zone. While a number of isolates group into identical VCGs, there is also a significant amount of genotypic diversity.

the mutualistic interaction between the plants and specific endophytes has been clearly demonstrated (Clay & Schardl 2002). The role of endophytes of woody plants is, however, much less clear. Opposing views have characterized them as mutualistic (Carroll 1988; Schardl 1996; Arnold *et al.* 2003), while others have viewed them as frequently neutral, or ranging from antagonistic to mutualistic (Ahlholm *et al.* 2002; Lehtonen *et al.* 2005; Faeth 2002; Faeth & Fagan 2002; Saikkonen *et al.* 1998; Saikkonen 2007; Sieber 2007).

The specific ecological role of the Botryosphaeriaceae is not clear for most of the species. Some Botryosphaeriaceae are well known as pathogens. This could imply that they are best viewed as pathogens with a latent phase. We would argue to the contrary. Many species exhibit only low or insignificant levels of pathogenicity when artificially inoculated (van Niekerk *et al.* 2004; Pavlic *et al.* 2007, personal observations). Such species are often also generally rare and are not commonly associated with diseases. Some well known pathogens on one host in a particular area might also not be very pathogenic to other hosts that they infect in other regions. For example, *B. dothidea* is one of the main pathogens of Pistachio and fruit trees in the USA (Ma *et al.* 2001, 2004; Michailides 1991), but is uncommon and appears only mildly pathogenic to healthy *Vitis*, *Eucalyptus* and *Syzygium* in South Africa (van Niekerk *et al.* 2004; Pavlic *et al.* 2007; Slippers *et al.* 2007). Here one must question what their ecological roles might be in natural communities where many of these 'non-pathogenic' isolates live in significant diversity. While no specific studies have been done on the potential beneficial or mutualistic interaction that Botryosphaeriaceae might have with plants, it would be worth examining this potential ecological role for the Botryosphaeriaceae based on studies of other known symbiotic fungal endophytes.

Carroll (1988, 1990) and other authors (see Saikkonen 2007 and Sieber 2007 for reviews) since have placed emphasis on the potential mutualistic protection of endophytes against insect damage. This is a common phenomenon in some grass-endophyte interactions (Clay 2001). Many have, however, disputed the common occurrence of this phenomenon in woody plants (Ahlholm *et al.* 2002; Faeth 2002; Saikkonen *et al.* 1998; Saikkonen 2007). In the case of the Botryosphaeriaceae, there is no evidence for the production of secondary metabolites that might directly affect herbivory (no study has, however, specifically searched for such compounds in these fungi) as is the case in some other endophytes (Clay & Schardl 2002; Petrini *et al.* 1992). Their biomass inside the healthy material also appears to be inordinately small to have an effect through such a direct mechanism (Smith 2001) (Figs 19, 20). However, they are likely to quickly colonize plant parts that have been damaged by insect herbivores. One consequence could be the dying off of such plant parts due to Botryosphaeriaceae, similar to a hypersensitive response. A branch infested by an insect, and dying off rapidly, could impair the completion of the life cycle of the insect by removing its food base, by competition with the mutualists of the insect, or by direct poisoning of the insects when they colonize the dying tissue. As example, such interactions have been shown to occur between *Phomopsis oblonga* and the scolytine vectors of the Dutch Elm Disease fungus, *Ophiostoma ulmi* (Webber 1981; Weber and Gibbs 1984; cited and discussed in Carroll 1990).

Another potential role for Botryosphaeriaceae is by speeding up the loss of redundant plant parts, specifically by killing or faster senescence of older or stressed leaves and branches, which are no longer contributing to photosynthesis (see also Carroll 1988 and Sieber 2007 regarding other fungi). This would release resources more efficient for new growth and reproduction. Furthermore, Carroll (1988) speculates that this process could lead to a build up of dry material that could fuel fires. This could be to the advantage of fire adapted species such as (i.e. Eucalypts and Proteaceae, both of which have common Botryosphaeriaceae endophytes which kill of branches and leaves (Denman *et al.* 2003; Slippers *et al.* 2004c; Swart *et al.* 2000).

Arnold *et al.* (2003) have shown a protective role of endophytes of tropical trees against a pathogen (*Phytophthora*). Carroll (1988) also reviewed other examples. No such studies have been conducted to determine potential antagonistic effects of Botryosphaeriaceae against other pathogens. However, the Botryosphaeriaceae in general compete very effectively on artificial medium for resources with most other fungi (personal observations during numerous isolations). Furthermore, when obligate parasites infect parts of the host, it is reasonable to speculate that Botryosphaeriaceae would rapidly colonize the stressed plant material, thereby potentially restricting the development of the other pathogen. This would correlate with the observation that Botryosphaeriaceae are often isolated from symptoms suspected to be caused by other pathogens (personal observations).

A number of studies suggest that endophytes of woody plants are rather loosely associated with their hosts, with higher correlation between endophyte communities in a specific location, than with a specific host in different locations (Arnold *et al.* 2003; Carroll & Carroll 1978; Fisher *et al.* 1993; Higgins *et al.* 2007; Saikkonen 2007). This is true also for the Botryosphaeriaceae. In agricultural and forestry situations, the introduced hosts frequently harbor Botryosphaeriaceae endophytes that are more similar to those on other hosts in that area, than those from the native range of the host. For example, *Eucalyptus* planted in different areas of the world harbor distinct Botryosphaeriaceae, some of which appear to be native to other plants and those areas rather than the native ranges of *Eucalyptus* (Burgess *et al.* 2005, 2006; Mohali *et al.* 2007; Slippers *et al.* 2004c; see discussions above). Furthermore, apple trees are commonly infected by *B. dothidea* in parts of the USA, but this fungal species is virtually absent in South African apple orchards. This is despite the fact that the fungus occurs in the area (Slippers *et al.* 2007). *Pinus radiata* appears to be exclusively infected by *D. scrobiculata* in its native range (California) (Burgess *et al.* 2004a), but in exotic environments (Australasia, Africa and South America) it is absent and the trees exclusively have endophytic infections of *D. pinea*, at a high frequency (Burgess *et al.* 2001a). These examples are based on extensive sampling efforts and detailed molecular studies (i.e not based on mistaken identities of the fungi). The studies have also not been based on single host species or cultivar in all locations, but many of the species mentioned appear to be particularly host specific.

The Botryosphaeriaceae is already remarkably species-rich, based on currently described species (see above 'Diversity, taxonomy and identification of Botryosphaeriaceae'). Recent studies in native ecosystems (ongoing projects where the authors are involved) and unique environments are revealing many unknown species (Burgess *et al.* 2005; Denman *et al.* 2003; Gure *et al.* 2005; Pavlic *et al.* 2004; Mohali *et al.* 2006), and it seems certain that such studies on a wider front are likely to reveal significantly greater diversity in the group. Furthermore, molecular tools are revealing previously overlooked cryptic diversity in established species (see above). The factors driving speciation in this group, however, have not been considered. Two main factors identified by Kohn (2005) that affect speciation in fungi, and that are likely to play a role for Botryosphaeriaceae as it relates their endophytic nature, are host range and dissemination. The Botryosphaeriaceae appear to have some level of host specificity, influenced by the host itself, or the host in a specific environment (i.e. some species with broad host ranges do not infect a known host in all locations where they co-occur; see discussion above). Furthermore, dissemination is likely to be restricted if the Botryosphaeriaceae is mostly rain-dispersed fungi. This factor will contribute to geographic population subdivision and ultimately allopatric speciation.

11. Summary and future work

Many species of the Botryosphaeriaceae are latent pathogens, especially in agricultural and forestry situations. However, very little is known regarding the role that most of these fungi play in native communities. Not even in intensively studied hosts, do we understand what the different ecological roles might be that the numerous species play when infecting the same host. It is not unlikely that some species might be mutualistic, as number of studies have demonstrated or suggested for other endophytes of woody hosts. Their common occurrence, potential threat as pathogens, ease of isolation, handling and sporulation in culture, their established taxonomic framework and the increasingly robust tools to study them, makes the Botryosphaeriaceae a useful and important model system to study a unique suite of endophytes. Such studies should contribute significantly to our understanding of endophyte-woody plant interactions.

A key shortcoming to our understanding of the Botryosphaeriaceae endophyte communities is that studies currently require isolation after surface sterilization. Thus, faster growing fungi, or fungi favored by artificial media will be over-represented. An obvious alternative is to apply modern detection tools to identify infection patterns, quantify levels of infection of different taxa, and thus to uncover hidden diversity. However, a key problem when using these tools is encountered when distinguishing between endophytic and epiphytic fungi. Studies are needed to develop tools that will allow the use of these modern molecular tools, with higher confidence that it will reflect the endophytic communities.

A platform for the taxonomy of the Botryosphaeriaceae has been provided in recent years. Numerous tools, particularly DNA-based molecular tools, have been developed to identify and describe species in the group. These tools should be applied to describe the diversity of the Botryosphaeriaceae as endophytes, especially in native plant communities, which have thus far been under sampled. An open question here is what factors have driven the extensive diversification in the group, a question to which the endophytic nature, ecological role, host affinity and geographic distribution is likely to be important factors for the group (see Kohn 2005).

All available evidence suggests that Botryosphaeriaceae are horizontally transmitted between individuals. However, the fact that they are also fairly commonly associated with seeds of some plants, raises the possibility of some level of vertical transmission. Such vertical transmissions can occur *via* systematic infection, or *via* asexual sporulation from a seed infection, followed by infection of the growing plant. Hand-in-hand with these studies will go a requirement to better understand the reproductive strategy, mode and distance of spread, predominant forms of infection (asexual or sexual) of the Botryosphaeriaceae (see Saikkonen *et al.* 1998). Answers to these questions will provide an important level of understanding regarding the ecological and evolutionary determinants of Botryosphaeriaceae-plant interactions.

One aspect that sets Botryosphaeriaceae apart from many other endophytes is the potential of some species to be aggressive pathogens when plants become stressed. They have also been moved extensively around the world, presumably due to their previously unknown endophytic nature. Indeed, they are most likely to continue to be moved widely given the lack of tools to detect them as latent infections, and the fact that planting stock continues to be extensively moved around the world. All evidence suggests that climate change will continue to increase stress on plant communities, and in this case some Botryosphaeriaceae are amongst the most likely fungi to cause wide scale damage. In the light of this threat, it is critical that we characterize changing interaction with plant communities and Botryosphaeriaceae under conditions imposed by climate change. Pathways by which they might spread need to be characterized and it is necessary to identify those species posing the greatest risk to plant communities. Furthermore, there is an urgent need to develop tools to rapidly and accurately detect these fungi *in planta*. Understanding the threat of especially 'host neutral' species, and the mechanisms that govern their infection, dispersal/spread and pathogenicity, will also be important for selecting, breeding or genetically manipulating plants for resistance to these intriguing and relatively poorly understood fungi.

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